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Two Distinct Pathways for Histamine H_2 Receptor Down-regulation

H_2 LEU^{124} → ALA RECEPTOR MUTANT PROVIDES EVIDENCE FOR A cAMP-INDEPENDENT ACTION OF H_2 AGONISTS*

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Pretreatment of Chinese hamster ovary cells expressing the histamine H_2 receptor ($CHOrH_2$ cells) with histamine resulted in a time-dependent ($t_{½} \approx 7$ h) and dose-dependent (EC_{50} = 18 nM) H_2 receptor down-regulation measured as [^{125}I]iodoaminopotentidine binding (44 ± 10% down-regulation). Pretreatment of $CHOrH_2$ cells with cholera toxin or forskolin also led to H_2 receptor down-regulation. Forskolin time-dependently ($t_{½} \approx 7$ h) and dose-dependently (EC_{50} = 0.3 μM) induced H_2 receptor down-regulation. Both histamine and forskolin induced rapid down-regulation of H_2 receptor mRNA levels, probably caused by mRNA destabilization.

Recently, Moro *et al.* (Moro, O., Lameh, J., Hogger, P., and Sadée, W. (1993) *J. Biol. Chem.* 268, 22273–22276) showed that hydrophobic amino acids in a conserved G-protein-coupled receptor motif in the second intracellular loop are implicated in G-protein coupling. To uncouple the H_2 receptor from the G_s-protein, we introduced the Leu^{124} → Ala mutation in the second intracellular loop of the H_2 receptor. The H_2 Leu^{124} → Ala mutant showed altered agonist-binding parameters, attenuated histamine-induced cAMP production, and was down-regulated by concentrations of histamine that did not give rise to cAMP production. Taken together, in $CHOrH_2$ cells, H_2 receptor down-regulation appears to be induced by two distinct pathways, a cAMP-dependent and cAMP-independent pathway.

The introduction of molecular biology in the field of histamine receptor research has greatly improved the possibilities to study molecular aspects of histamine receptor proteins. In 1991, Gantz *et al.* (1) cloned the cDNA encoding the canine histamine H_2 receptor, which was followed by the cloning of both the rat and human homologues (2, 3). The deduced amino acid sequence of the H_2 receptor proteins reveals the existence of seven putative transmembrane domains, indicating that this receptor is a member of the large family of G-protein-coupled receptors (GPCR).[1] This family of receptors is known to be readily subjected to regulatory processes in order to control receptor signaling and thus cellular communication (4). Short-term exposure of receptors to high concentrations of agonists is often followed by a decrease in cellular responsiveness, called desensitization (5). Long-term exposure, on the other hand, results in the reduction of receptor number (6) and is referred to as receptor down-regulation. Since the histamine H_2 receptor is a member of this family of GPCRs, it is not surprising that this receptor is also susceptible to such regulatory mechanisms.

Recently, we have shown that in human U937 cells the endogenously expressed histamine H_2 receptors are indeed rapidly desensitized when exposed to histamine (7). Similar observations have been reported in other cellular systems (8, 9). Yet, so far, no detailed information is available on long-term desensitization of the histamine H_2 receptor such as receptor down-regulation. Such processes may become apparent under several pathophysiological conditions (*e.g.* asthmatic attack or allergic reactions in general), during which histamine is released in large quantities, but might also occur under normal physiological conditions. Recently, Diaz *et al.* (10) suggested for example that *in vivo* receptor down-regulation might explain the inverse relationship between H_2 receptor expression and the localization of histamine-synthesizing cells in the rodent gastric wall. The regulation of H_2 receptor expression has gained further interest due to the potential therapeutic application of H_2 receptor agonists in patients suffering from congestive heart failure (11).

Investigation of the regulation of H_2 receptor expression has so far been hampered by the availability of suitable model systems. Cellular systems (7–9, 12) have been used to investigate second messenger responses coupled to the histamine H_2 receptor stimulation, but the used systems such as U937 cells for example do not express a sufficiently high density of H_2 receptors to permit radioligand binding studies, which are essential for the investigation of long-term regulatory mechanisms (7). Following the recent cloning of cDNAs or genes encoding histamine H_2 receptors, cell lines expressing considerable amounts of histamine H_2 receptors can be obtained (13, 14). Additionally, the availability of the H_2 receptor gene allows the construction of receptor mutants, which can provide mechanistic insights in phenomena like receptor down-regulation.

In the present study we have examined the effects of long-term exposure of the rat histamine H_2 receptor stably expressed in Chinese hamster ovary (CHO) cells (referred to as $CHOrH_2$ cells) (13) to H_2 agonists and cAMP mobilizing agents with regard to H_2 receptor protein expression and H_2 receptor mRNA levels. In order to get more insight into the mechanisms underlying H_2 receptor regulation, we constructed a H_2 receptor mutant, in which leucine 124 in the second intracellular

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This paper is dedicated to Prof. Dr. E. Mutschler on the occasion of his 65th birthday.

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[1] The abbreviations used are: GPCR, G-protein-coupled receptor; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; [^{125}I]APT, [^{125}I]iodoaminopotentidine; IBMX, isobutylmethylxanthine; CTX, cholera toxin; PCR, polymerase chain reaction; GTPγS, guanosine 5′-*O*-(thiotriphosphate).

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loop was substituted by an alanine. This H_2 $Leu^{124} \rightarrow Ala$ receptor mutant was partially uncoupled from its G-protein and proved to be a suitable tool for investigating the existence of possible cAMP-dependent and independent pathways in the process of agonist-induced H_2 receptor down-regulation.

MATERIALS AND METHODS

Cell Culture—CHO cells expressing the rat histamine H_2 receptor ($CHOrH_2$) (13) and the mutated H_2 $Leu^{124} \rightarrow Ala$ receptor ($CHOrH_2Leu^{124}Ala$) were grown at 37 °C in a humidified atmosphere with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM), containing 10% (v/v) dialyzed fetal calf serum supplemented with 2 mM L-glutamine, MEM amino acids, 50 IU/ml penicillin, and 50 μg/ml streptomycin.

Site-directed Mutagenesis—The H_2 receptor mutant, in which leucine 124 was substituted by alanine ($Leu^{124} \rightarrow Ala$) (Fig. 7), was constructed by means of the polymerase chain reaction. The oligonucleotides S1 (5′-GGGAAGCTTGGCCCCAGAATGGAGCCCAATGGCACAGT), corresponding to nucleotides −9 to 21 (2) and a *Hin*dIII site (underlined), and AS1 (5′-GGGGGTACCGCGCTGGGTCCGTGACAGCGCAGTAG-TTGTTCAAGCTGATCAT), corresponding to nucleotides 358 to 383 of the complementary strand (2) containing a *Kpn*I site (underlined) with two nucleotide changes, were synthesized on an Applied Biosystems DNA synthesizer (model 381A). Using 100 ng of $pSVrH_2$ (13) as a template, 0.4 μM S1, 0.4 μM AS2, 40 μM dNTPs, and 2.5 units of Amplitaq according to the manufacturer's protocol (Perkin Elmer), a 392-base pair DNA fragment of the H_2 $Leu^{124} \rightarrow Ala$ receptor mutant was amplified in 100 μl using 30 cycles at 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 1.5 min and a final extension at 72 °C for 10 min. The obtained PCR product was gel-purified and restricted with *Hin*dIII/*Kpn*I (Boehringer). This fragment was cloned into the plasmid pSP73 (Promega) containing the wild-type rH_2 receptor, which was restricted with *Kpn*I and *Hin*dIII. Thereafter, the PCR-amplified sequence was verified using the dideoxy chain termination method with the Sequenase kit (U. S. Biochemical Corp.). Subsequently, the coding sequence of the mutated H_2 $Leu^{124} \rightarrow Ala$ receptor was subcloned into the eukaryotic pSV expression vector. CHO cells, deficient in dihydrofolate reductase, were stably transfected with 15 μg of $pSVrH_2Leu^{124}Ala$ using Transfectam (Promega).

Membrane Preparation—$CHOrH_2$ and $CHOrH_2Leu^{124}Ala$ cells were washed three times with cold phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4) and harvested with a cell scraper and recovered by a 10-min centrifugation at 500 × *g*. Cells were homogenized in ice-cold 50 mM Na_2/potassium phosphate buffer (pH 7.4) with a Polytron homogenizer (5 s, maximal speed) and used for radioligand binding studies. Protein concentrations were determined according to Bradford using bovine serum albumin as a standard (15).

Histamine H_2 Receptor Binding—The radiolabeled H_2 receptor antagonist [^{125}I]iodoaminopotentidine ([^{125}I]APT) was synthesized as described previously (14). Triplicate assays were performed in polyethylene tubes in 50 mM Na_2/potassium phosphate buffer containing gelatin (0.1%) to prevent adsorption of the radioligand. In saturation studies, increasing concentrations of [^{125}I]APT were incubated with 5–10 μg of membrane proteins in the absence or presence of 1 μM tiotidine in a total volume of 400 μl. After 90 min at 30 °C, the incubations were stopped by rapid dilution with 3 ml of ice-cold 20 mM Na_2/potassium phosphate buffer (pH 7.4) supplemented with 0.1% bovine serum albumin. The bound radioactivity was subsequently separated by filtration with a Brandel cell harvester (Semat) through Whatman GF/B glass fiber filters that had been treated with 0.3% polyethyleneimine. Filters were washed twice with 3 ml of buffer, and radioactivity retained on the filters was counted with a LKB-γ-counter at an efficiency of 63%. The binding data were evaluated by use of LIGAND, a nonlinear, weighted, least squares curve-fitting procedure (16). Changes in H_2 receptor density were denoted as a percentage down-regulation compared to non-treated control cells. During the 24-h incubation of cells with various histamine ligands or other compounds, cells were maintained in medium without fetal calf serum.

Cyclic AMP Production—$CHOrH_2$ and $CHOrH_2Leu^{124}Ala$ cells were seeded in 24-well plates and cultured overnight in culture medium. Cells were washed twice with DMEM, supplemented with 50 mM HEPES (pH 7.4 at 37 °C), and preincubated for 30 min at 37 °C. Thereafter, the medium was aspirated, appropriate drugs in DMEM/HEPES supplemented with 300 μM phosphodiesterase inhibitor isobutylmethylxanthine (IBMX) were added, and the cells were incubated for 10 min at 37 °C. The reaction was stopped by the rapid aspiration of the culture medium and the addition of 200 μl of 0.1 N cold HCl. The cells were kept on ice and disrupted by sonification (5 s, 50 watts, Labsonic 1510, Braun-Melsungen). The resulting homogenate was frozen at −20 °C or directly neutralized with 1 N NaOH and assayed for the presence of cAMP. In order to determine the long-term effects of histamine treatment on H_2 receptor signaling, $CHOrH_2$ cells were preincubated with 100 μM histamine for 24 h in DMEM without fetal calf serum. Thereafter, these cells were thoroughly washed and preincubated for 1 h in DMEM/HEPES at 37 °C before actual incubation with the indicated drugs.

Cyclic AMP Assay—The amount of cAMP in the $CHOrH_2$ and $CHOrH_2Leu^{124}Ala$ cells was determined according to Nordstedt and Fredholm (17), with some minor modifications. Briefly, a protein kinase A-containing fraction was isolated from bovine adrenal glands. Adrenal cortex was homogenized in 10 volumes of 100 mM Tris-HCl, 250 mM NaCl, 10 mM EDTA, 0.25 M sucrose, and 0.1% 2-mercaptoethanol (pH 7.4 at 4 °C, buffer A) using an Omni-Sorval mixer (30 s, maximal speed) and a Polytron homogenizer (10 s, maximal speed). The homogenate was centrifuged for 60 min at 30,000 × *g* at 4 °C. The supernatant, containing protein kinase A, was carefully recovered and frozen in 1 ml aliquots at −80 °C. Before use, the binding protein was diluted 5-fold in ice-cold buffer A without sucrose and 2-mercaptoethanol and kept on ice. Subsequently, 200 μl of the binding protein was mixed with 50–100 μl of the CHO homogenate or cAMP standards and 30,000 dpm of [^{3}H]cAMP. After incubation for 150 min at 4 °C, the mixture was rapidly diluted with 3 ml of ice-cold 50 mM Tris-HCl (pH 7.4 at 4 °C) and filtered through Whatman GF/B filters using a Brandel cell-harvester (Semat). The radioactivity retained on the filters was measured by liquid scintillation counting.

RNA Slot Blot Analysis—RNA was analyzed by means of mRNA slot blot assay as described by Zhang *et al.* (18), with minor modifications. Briefly, RNA was isolated according to the method of Chomczynski and Sacchi (19), using Trizol reagent® (Life Technologies, Inc.), and RNA was slot-blotted on a nitrocellulose filter (GeneScreen Plus, DuPont NEN) and prehybridized for 2 h at 65 °C in 7% SDS, 0.5 M Na_2HPO_4, 1 mM EDTA (pH 7.2). Thereafter, filters were hybridized overnight with a radioactive labeled 48-mer antisense rat H_2 receptor oligonucleotide (5′-GATGGTGGCTGCCTTCCAGGAGCTGATGTGGTTGATCCGTT-TGGCCTG-3′, corresponding to nucleotides 631 to 678) or antisense rat β-actin oligonucleotide (5′-CTCCTGCTTGCTGATCCACATCTGCTGG-AAGGTGGACAGTGAGGCCAG-3′, corresponding to nucleotides 3040 to 3087) at 65 °C in 7% SDS, 0.5 M Na_2PO_4, and 1 mM EDTA (pH 7.2). The rat H_2 receptor oligonucleotide (1.5 pmol) was ^{32}P-labeled by 3′-end tailing using 16 pmol of [α-^{32}P]dATP (3000 Ci/mmol; Amersham) and 1 unit of terminal deoxynucleotidyltransferase (Boehringer) for 20 min at 37 °C. The β-actin oligonucleotide (5 pmol) was labeled using 10 pmol of [γ-^{32}P]dATP (3000 Ci/mmol; Amersham) and 4 units of polynucleotide kinase (Boehringer) for 30 min at 37 °C. The blots were washed twice for 5 min at room temperature in 2 × SSC (0.3 M NaCl, 0.03 M $Na_3C_6H_5O_7 \cdot 2H_2O$) supplemented with 0.1% SDS, which was followed by two 45-min washes at 65 °C with 2 × SSC supplemented with 0.1% SDS. The blots were exposed to a PhosphorScreen (Molecular Dynamics) and signals were quantified with a PhosphorImager 425 (Molecular Dynamics) using the computer program ImageQuant (Molecular Dynamics). H_2 receptor mRNA levels were expressed as the ratio of the values of the H_2 receptor mRNA signals and the corresponding β-actin signals.

Analysis of H_2 Receptor mRNA Stability—H_2 receptor mRNA levels were determined after incubation of the $CHOrH_2$ cells with actinomycin D to block transcription as described previously (20). Cells were preincubated with or without 100 μM histamine or with 10 μM forskolin for 1 h in DMEM. Thereafter, actinomycin D (10 μg/ml) was added. Cells were harvested from 0 to 90 min after addition of actinomycin D. Total RNA was extracted at each time point, and H_2 receptor mRNA was quantified by means of the mRNA slot blot assay as described above.

Chemicals—Histamine dihydrochloride, isobutylmethylxanthine (IBMX), cyclic AMP (cAMP), forskolin, 1,9-dideoxyforskolin, cholera toxin, and GTPγS were obtained from Sigma. Actinomycin D was purchased from Boehringer Mannheim. [2,8-^{3}H]cAMP (40 Ci/mmol) was obtained from Amersham. H-89 dihydrochloride and KT5720 were purchased from Calbiochem. Dimaprit dihydrobromide, homo- and nordimaprit dihydrochloride, amthamine dihydrobromide, amselamine dihydrobromide, and aminopotentidine were taken from laboratory stock. Gifts of cimetidine (SmithKline Beecham), ranitidine dihydrochloride (Glaxo), tiotidine (Imperial Chemical Industries), CHO cells expressing the rat H_2 receptor, and $pSVrH_2$ vector (Dr. J.-C. Schwartz) are gratefully acknowledged.

Statistical Analysis—All data shown are expressed as mean ± S.E. of at least three independent experiments. Statistical analysis was carried

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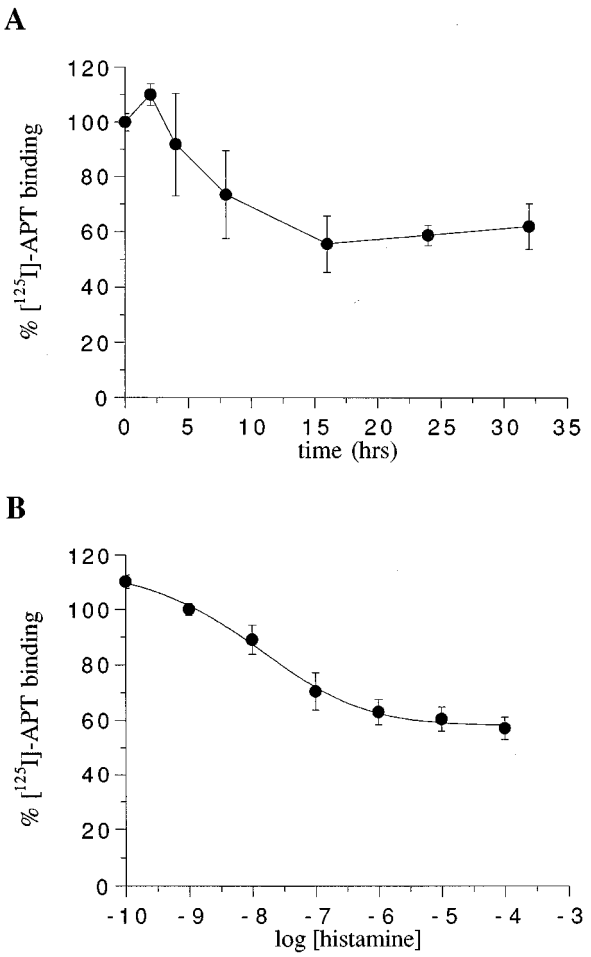

FIG. 1. **Time- and dose-dependent decrease of [^{125}I]APT binding in CHOrH_2 cells by histamine.** *A*, CHOrH_2 cells were incubated with 100 μM histamine for the indicated times, and [^{125}I]APT binding in membranes was measured. The [^{125}I]APT binding is expressed as a percentage of [^{125}I]APT binding measured in nontreated cells. The data shown represent the mean ± S.E. of 4 independent experiments. *B*, dose-dependent decrease of [^{125}I]APT binding induced by histamine. CHOrH_2 cells were exposed to various concentrations of histamine for 24 h. The data represent the mean ± S.E. of 7 independent experiments.

out by Student's *t*-test. p values < 0.05 were considered to indicate a significant difference.

RESULTS

Histamine-induced H_2 Receptor Down-regulation—Exposure of CHOrH_2 cells (13) to 100 μM histamine for prolonged periods of time resulted in a time-dependent decrease of [^{125}I]APT binding (Fig. 1*A*). Maximum reduction of [^{125}I]APT binding was observed after a 16-h incubation of cells with 100 μM histamine. Under this condition, histamine induced 44 ± 10% ($p < 0.05$) reduction of [^{125}I]APT binding. Half-maximum reduction of the [^{125}I]APT binding was recorded at an incubation period of approximately 7 h. A 24-h incubation of CHOrH_2 cells with increasing concentrations of histamine led to a dose-dependent reduction of [^{125}I]APT binding (EC_{50} value = 18 ± 6 nM, mean ± S.E., n = 7) (Fig. 1*B*). The observed reduction of [^{125}I]APT binding was not reflected by a change in affinity of [^{125}I]APT for the H_2 receptor, as its dissociation constant (K_d), determined by means of saturation studies, remained unaffected in CHOrH_2 cells incubated with 100 μM histamine for 24 h (Table I). Exposure of CHOrH_2 cells to histamine resulted only in a marked decrease of the total number of [^{125}I]APT binding sites (B_{max}) (Table I).

The recently described selective H_2 receptor agonists amselamine and amthamine (21, 22) induced cAMP production in CHOrH_2 cells, with EC_{50} values lower and maximum responses comparable to histamine (Table II). Long-term exposure (24 h) of CHOrH_2 cells to amselamine or amthamine resulted in a dose-dependent decrease of [^{125}I]APT binding with EC_{50} values, which were correlated with their respective EC_{50} values for the cAMP response. Amselamine and amthamine induced a maximal decrease of [^{125}I]APT binding sites of 50 ± 3% and 43 ± 4% respectively. As shown in Table II, 24-h incubation of CHOrH_2 cells with 100 μM dimaprit, which exhibits a lower potency as compared to histamine, induced a 40 ± 3% decrease of [^{125}I]APT binding sites. Dimaprit's structural analogues, homodimaprit and nordimaprit, showed strongly reduced capacities to generate cAMP with EC_{50} values of 1.4 ± 0.9 μM and higher than 10 μM, respectively (Table II). The reduced ability of these dimaprit analogues to induce a cAMP response was paralleled by a lack of H_2 receptor down-regulation after 24 h of incubation of CHOrH_2 cells with 100 μM concentrations of the analogues (Table II).

Effect of Long-term Histamine Treatment on Histamine- and Forskolin-induced Signaling in CHOrH_2 Cells—Long-term exposure (24 h) of CHOrH_2 cells with 100 μM histamine resulted in a rightward shift of the dose-response curve of the histamine-induced cAMP production (EC_{50} of histamine-induced cAMP response in nontreated cells: 36 ± 3 nM, mean ± S.E., n = 7, and histamine-treated cells: 1.2 ± 0.05 μM, mean ± S.E., n = 4) (Fig. 2*A*). The forskolin-induced rise in cAMP was not found to be affected as no change in dose dependence or im-

TABLE I

Characteristics of the [^{125}I]APT binding to the rat histamine H_2 receptor expressed in CHO cells pretreated for 24 h with or without histamine or forskolin

The dissociation constant (K_d) and maximum number of binding sites (B_{max}) were determined using nonlinear fitting according to a one-site binding mode. The data shown represent the mean ± S.E. of 3 independent experiments.

Pretreatment (24 h)	[^{125}I]APT binding	
	K_d	B_{max}
	nM	*fmol/mg protein*
Control	0.43 ± 0.06	975 ± 12
Histamine (100 μM)	0.42 ± 0.05	468 ± 129[a]
Forskolin (10 μM)	0.29 ± 0.03[a]	324 ± 59[a]

[a] Significant difference ($p < 0.05$) from control, represented by untreated cells.

TABLE II

EC_{50} values and the percentage of maximally induced production (E_{max}) of different H_2 agonists for production of cAMP and the maximally induced effect of a 24-h incubation of these compounds on [^{125}I]APT binding in CHOrH_2 cells

Data of the E_{max} values are shown as a percentage of the histamine (100 μM)-induced cAMP response. The effect of 24 h of incubation of CHOrH_2 cells with the different compounds on [^{125}I]APT binding is expressed as the percentage of H_2 receptor down-regulation. The data shown represent the mean ± S.E. of 4 to 8 independent experiments.

	cAMP response		Down-regulation	
	EC_{50}	% E_{max}	EC_{50}	% receptor down-regulation
Histamine	66 ± 29 nM	100 ± 4	18 ± 6 nM	41 ± 6[a]
Amselamine	15 ± 6 nM	88 ± 10	5 ± 1 nM	50 ± 3[a]
Amthamine	5 ± 1 nM	89 ± 6	2 ± 1 nM	43 ± 4[a]
Dimaprit	0.2 ± 0.07 μM	122 ± 8	0.1 ± 0.04 μM	40 ± 3[a]
Nordimaprit	>10 μM	70 ± 3[b]	>10 μM	7 ± 9[c]
Homodimaprit	1.4 ± 0.9 μM	38 ± 1	>10 μM	11 ± 5[c]

[a] Significant difference ($p < 0.05$) from control, represented by nontreated cells.
[b] The E_{max} value of nordimaprit was determined using a concentration of 1 mM.
[c] Determined at 100 μM.

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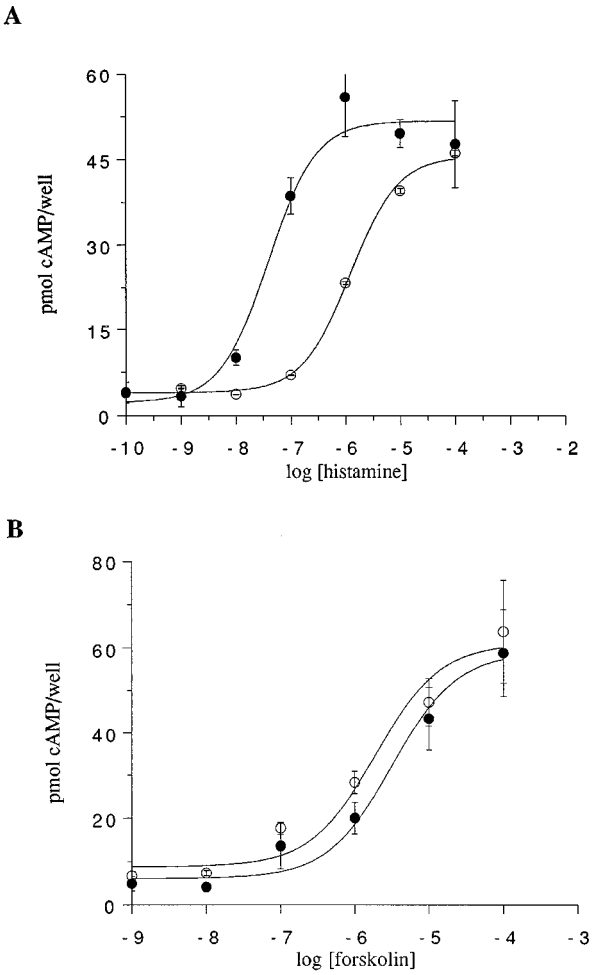

FIG. 2. **Effect of long-term histamine treatment on histamine- and forskolin-induced signaling in $CHOrH_2$ cells.** $CHOrH_2$ cells were treated with (*open circles*) or without (*filled circles*) 100 μM histamine for 24 h in DMEM without fetal calf serum. Thereafter, cells were washed several times and incubated for 1 h with DMEM supplemented with 25 mM HEPES, pH 7.4. $CHOrH_2$ cells were subsequently incubated with increasing concentrations of histamine (*A*) or forskolin (*B*) for 10 min at 37 °C in DMEM in the presence of 300 μM IBMX and 25 mM HEPES, pH 7.4. The data represent the mean ± S.E. of 4 independent experiments.

pairment of the maximal forskolin-induced cAMP response was observed after a 24-h pretreatment of cells with 100 μM histamine (Fig. 2*B*).

Role of cAMP in the Process of H_2 Receptor Down-regulation—Forskolin, which directly activates adenylyl cyclase, dose dependently induced the formation of cAMP in $CHOrH_2$ cells (Fig. 3*A*). Prolonged exposure (incubation periods ranging from 4 to 32 h) of $CHOrH_2$ cells with 10 μM forskolin led to a marked reduction of 58 ± 2% [^{125}I]APT binding (Fig. 3*B*). Again, no major change in affinity of [^{125}I]APT for the H_2 receptor was apparent, only a decrease in B_{max} was observed when $CHOrH_2$ cells were incubated for 24 h with 10 μM forskolin (Table I). Maximum and half-maximum down-regulation was recorded after 16 h and approximately 7 h of incubation of $CHOrH_2$ cells with 10 μM forskolin, respectively (Fig. 3*B*). The H_2 receptor binding sites appeared to be dose dependently down-regulated by increasing concentrations of forskolin, with an EC_{50} value of 0.3 ± 0.06 μM (mean ± S.E., $n = 4$) (Fig. 3*C*). Concentrations up to 10 μM of the inactive analogue 1,9-dideoxyforskolin, which does not generate cAMP in $CHOrH_2$ cells (Fig. 3*A*), did not attenuate the H_2 receptor density after 24 h of pretreatment (Fig. 3*D*).

Cholera toxin (CTX), which irreversibly activates the G_s-protein, thereby generating cAMP, also induced a dose-dependent decrease of [^{125}I]APT binding sites when incubated for 24 h (EC_{50} = 32 ± 1 ng/ml, mean ± S.E., $n = 4$, Fig. 4). CTX pretreatment of $CHOrH_2$ cells resulted in a maximum down-regulation of H_2 receptors of 46 ± 3%. Finally, exposure of $CHOrH_2$ cells for 24 h to 300 μM IBMX, a cAMP phosphodiesterase inhibitor, also resulted in an attenuation of [^{125}I]APT binding (26 ± 7% H_2 receptor down-regulation, $n = 4$, mean ± S.E., $p < 0.05$).

H_2 Receptor mRNA Levels and Stability in Control, Histamine-treated, and Forskolin-treated $CHOrH_2$ Cells—Exposure of $CHOrH_2$ cells to 100 μM histamine for increasing periods of time resulted in a rapid transient decrease of H_2 receptor mRNA (maximum reduction of 71 ± 4%, mean ± S.E., $n = 4$) (Fig. 5). This effect was at its peak after 4 h of incubation of cells with histamine (100 μM), while the amount of H_2 receptor mRNA returned to approximately 50% of control after 12 h of histamine treatment. Long-term incubation of $CHOrH_2$ cells with 10 μM forskolin also induced a time-dependent transient decrease (maximum reduction: 75 ± 7%, mean ± S.E., $n = 4$) of H_2 receptor mRNA to levels similar to those observed after histamine treatment (Fig. 5).

To study the role of mRNA stability, $CHOrH_2$ cells were incubated for 1 h in the absence or presence of histamine (100 μM) or forskolin (10 μM), whereafter actinomycin D (10 μg/ml) was added to block mRNA transcription. Cells were collected at different time intervals ranging from 0 to 90 min after addition of actinomycin D and were analyzed for H_2 receptor mRNA content. The H_2 receptor mRNA in nontreated cells was hardly affected during the 90 min of incubation with actinomycin D (*inset*, Fig. 5). Incubation of cells with 100 μM histamine, however, resulted in a significant breakdown of H_2 receptor mRNA levels (*inset*, Fig. 5). Similar results were obtained after forskolin treatment (*inset*, Fig. 5).

Differences between Histamine- and Forskolin-induced H_2 Receptor Down-regulation—As can be seen in Fig. 5, 4 h of incubation of cells with 100 μM histamine resulted in a marked down-regulation (71 ± 4%) of the H_2 receptor mRNA, whereas after 4 h of incubation of $CHOrH_2$ cells and direct measurement of [^{125}I]APT binding, no significant down-regulation of H_2 receptors was observed (Fig. 1*A*). Yet, when $CHOrH_2$ cells were incubated with 100 μM histamine for 1, 2, or 4 h, extensively washed and further incubated in serum-free medium without histamine for 23, 22, and 20 h, respectively, a significant reduction of H_2 receptor binding sites was observed (Fig. 6*A*). Interestingly, similar experiments in which $CHOrH_2$ cells were incubated for 1, 2, or 4 h with 10 μM forskolin followed by extensive washing and further incubation of cells in serum-free medium, showed a clearly delayed reduction in the number of H_2 receptor binding sites (Fig. 6*A*).

In order to eliminate the cAMP-dependent pathway of H_2 receptor down-regulation, we incubated the $CHOrH_2$ cells with the protein kinase A inhibitor H-89. However, long-term exposure (24 h) of $CHOrH_2$ cells to 10 μM H-89 resulted already in a 55 ± 2% (mean ± S.E., $n = 6$) decrease of [^{125}I]APT binding sites. Similar data were obtained with another protein kinase A inhibitor KT5720.[2] Taking into account that H-89 itself induces a reduction in [^{125}I]APT binding sites, $CHOrH_2$ cells were exposed to 1 μM histamine and 1 μM forskolin in the presence of 10 μM H-89 for 24 h. As can be seen in Fig. 6*B*, the forskolin-induced effect is inhibited by co-incubation with 10 μM H-89 as no H_2 receptor down-regulation is observed. Yet, long-term incubation of cells with histamine and H-89 still

[2] M. J. Smit, unpublished observations.

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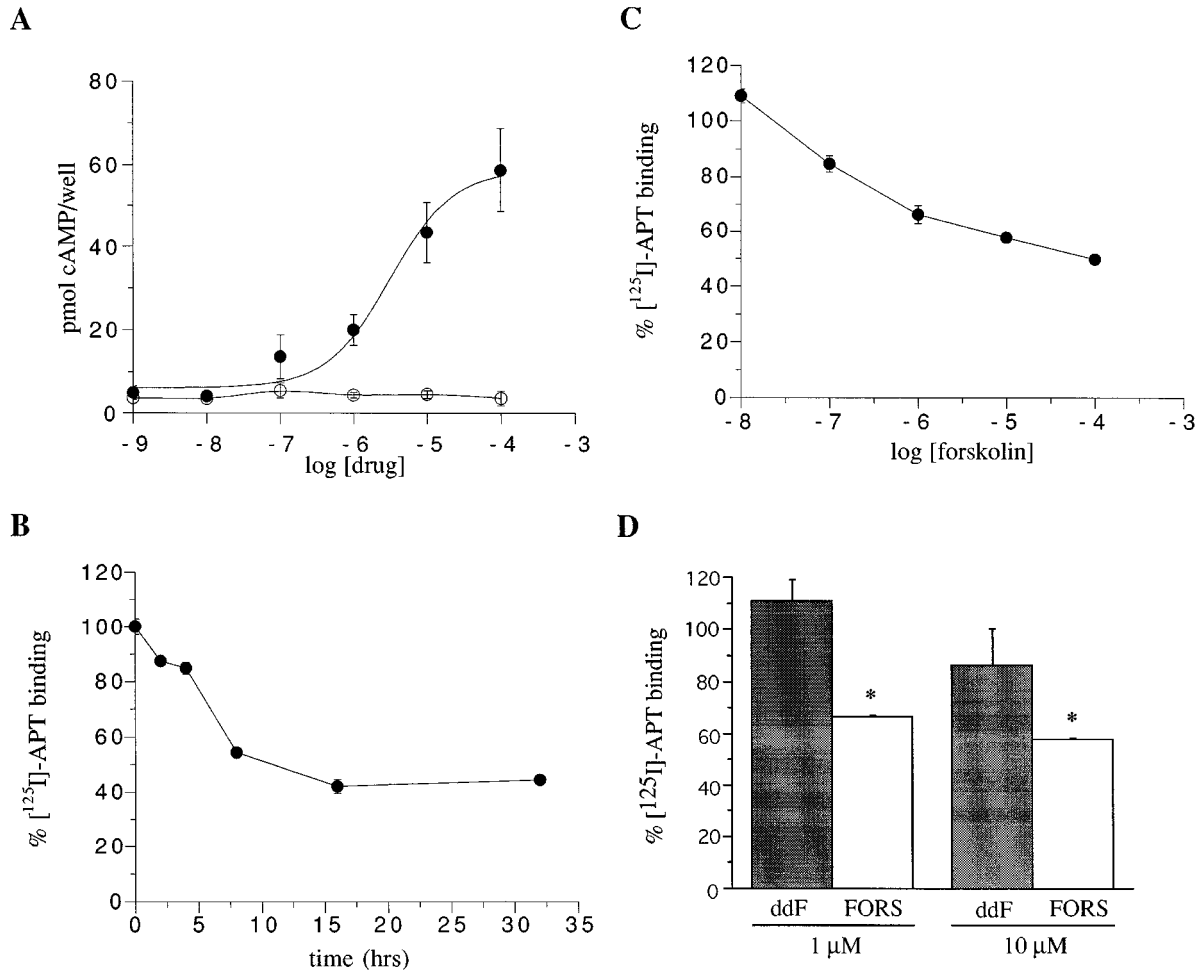

FIG. 3. **Forskolin- and 1,9-dideoxyforskolin-induced cAMP production and decrease of [^{125}I]APT binding in CHOrH_2 cells.** *A*, dose-dependent increase of the cAMP production by forskolin (*filled circles*) and 1,9-dideoxyforskolin (*open circles*). CHOrH_2 cells were incubated with the indicated drugs with increasing concentrations for 10 min at 37 °C in DMEM in the presence of 300 μM IBMX and 25 mM HEPES, pH 7.4. Data represent the mean ± S.E. of 6 independent experiments. *B*, forskolin-induced decrease of [^{125}I]APT binding in CHOrH_2 cells. CHOrH_2 cells were incubated with 10 μM forskolin for the indicated times, and [^{125}I]APT binding was measured. The [^{125}I]APT binding is expressed as a percentage of [^{125}I]APT binding measured in nontreated cells. *C*, dose-dependent decrease of [^{125}I]APT binding in CHOrH_2 cell membranes induced by forskolin. CHOrH_2 cells were exposed to various concentrations of forskolin for 24 h. The H_2 receptor density was determined as described. *D*, effect of 1,9 dideoxyforskolin (*ddF*) and forskolin (*FORS*) on [^{125}I]APT binding. CHOrH_2 cells were exposed to 1 and 10 μM 1,9-dideoxyforskolin (*filled bars*) and forskolin (*open bars*), respectively, for 24 h and examined for [^{125}I]APT binding. The *asterisks* indicate a significant difference ($p < 0.05$) from control, represented by the [^{125}I]APT binding measured in untreated cells. Data from *B*, *C*, and *D* were calculated as mean ± S.E. from 4 independent experiments.

induces down-regulation, suggesting that a cAMP-independent pathway is responsible for the histamine-induced down-regulation.

Functional Analysis of the Leu^{124} → Ala Mutation of the Rat Histamine H_2 Receptor—Using the polymerase chain reaction, leucine 124 in the second intracellular loop of the rat histamine H_2 receptor (Fig. 7) was mutated into an alanine residue. Transfection of the H_2 Leu^{124} → Ala receptor cDNA into CHO cells, resulted in the formation of several clonal cell lines expressing [^{125}I]APT binding sites. A clonal cell line expressing amounts of [^{125}I]APT binding comparable to those of the CHOrH_2 cells was chosen for further analysis and referred to as CHOrH_2Leu^{124}Ala (CHOrH_2 cells: 975 ± 12 fmol/mg of protein, CHOrH_2Leu^{124}Ala cells: 980 ± 7 fmol/mg of protein, mean ± S.E., n = 3). There were no major differences in the binding of the H_2 antagonists to the wild-type receptor or the mutated receptor. The affinity of [^{125}I]APT for the mutated receptor was hardly affected (K_d of [^{125}I]APT in CHOrH_2 cells: 0.43 ± 0.06 nM, in CHOrH_2Leu^{124}Ala cells: 0.61 ± 0.03 nM, mean ± S.E., n = 3). Moreover, cimetidine and ranitidine had similar K_i values for both receptors (Table III). In contrast, the introduced Leu^{124} → Ala mutation significantly affected the agonist binding characteristics. In CHOrH_2 cells, histamine displacement curves were shallow and could be analyzed best by a two-site model (Fig. 8, Table III). The addition of 10 μM GTPγS resulted in a steepening and a rightward shift of the histamine displacement curve, which could be analyzed best by a single site model with a K_i value of 0.18 ± 0.02 mM (Fig. 8). In CHOrH_2Leu^{124}Ala cells, however, the displacement curve of histamine was analyzed best by a single site model, leading to a K_i value (0.21 ± 0.02 mM) that corresponded to the low affinity site of the wild-type receptor (Fig. 8, Table III). The addition of 10 μM GTPγS did not result in a rightward shift of the displacement curve of histamine (Fig. 8, Table III).

Moreover, the Leu^{124} → Ala mutation also affected the ability of histamine to induce the formation of cAMP in CHOrH_2Leu^{124}Ala cells (Fig. 9*A*). The EC_{50} value of the histamine-induced cAMP response in CHOrH_2Leu^{124}Ala cells was approximately 162-fold higher (11 ± 3 μM, mean ± S.E., n = 7) than the observed EC_{50} value of the histamine-induced cAMP response in CHOrH_2 cells (66 ± 29 nM, mean ± S.E., n = 6) measured under the same conditions. The maximum hista-

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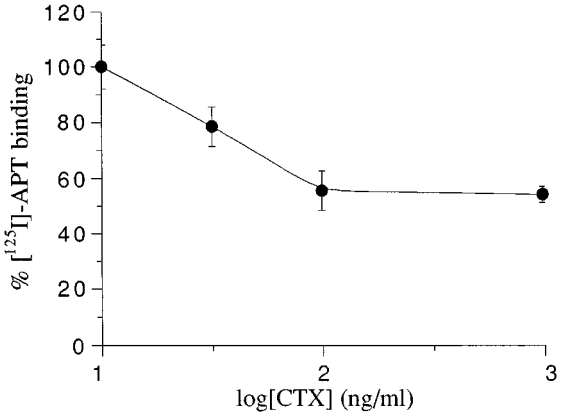

FIG. 4. **Effect of long-term treatment with CTX on [^{125}I]APT binding in CHOrH_2 cells.** The CHOrH_2 cells were treated with increasing concentrations of CTX for 24 h. [^{125}I]APT binding is expressed as a percentage of [^{125}I]APT binding measured in nontreated cells studied under the same conditions and was measured as described earlier. The data shown represent the mean ± S.E. of 4 independent experiments.

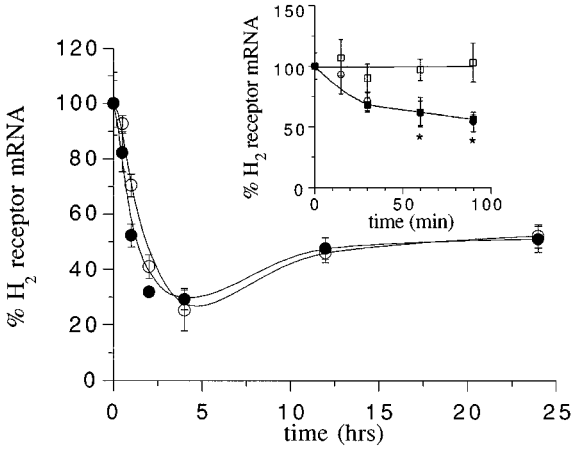

FIG. 5. **Histamine- and forskolin-induced modulation of H_2 receptor mRNA levels.** CHOrH_2 cells were incubated for the indicated times with 100 μM histamine (*filled circles*) or 10 μM forskolin (*open circles*). Cells were harvested, and total RNA was extracted and quantified by means of a RNA slot blot assay as described under "Materials and Methods." The results displayed are the mean ± S.E. of two separate experiments, performed in duplicate. *Inset*, effect of histamine treatment and forskolin treatment on H_2 receptor mRNA stability. CHOrH_2 cells were incubated with (*open circles*) or without (*open squares*) 100 μM histamine or with 10 μM forskolin (*filled squares*) for 1 h, before actinomycin D (10 μg/ml) was added. Cells were harvested at 0, 15, 30, 60, and 90 min after the addition of actinomycin D. The data are the mean ± S.E. of three separate experiments, each performed in duplicate. The *asterisks* indicate a significant difference ($p < 0.05$) from control, represented by untreated cells.

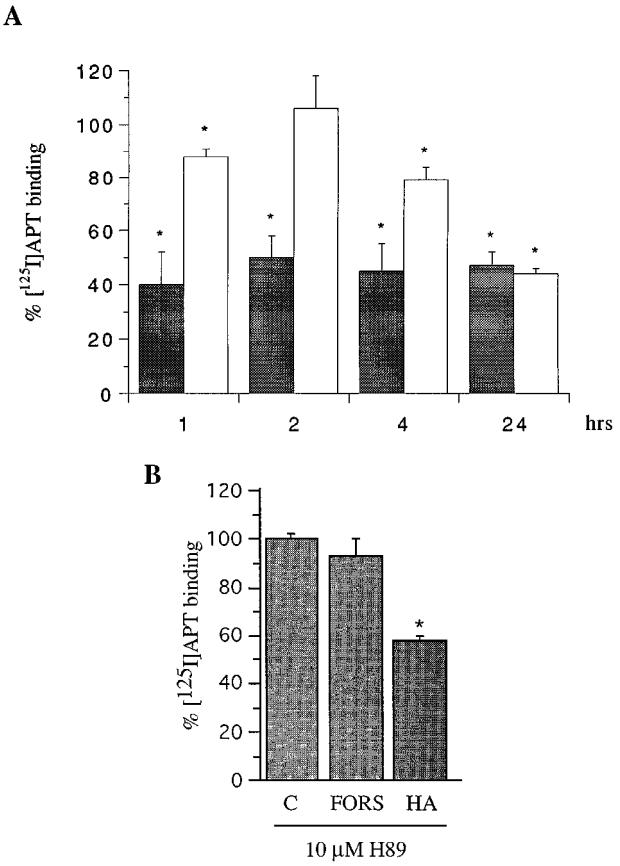

FIG. 6. **Differences between histamine- and forskolin-induced H_2 receptor down-regulation.** *A*, effect of short-term exposure of CHOrH_2 cells to histamine and forskolin on [^{125}I]APT binding to CHOrH_2 membranes. CHOrH_2 cells were incubated for the indicated times, extensively washed, and incubated in serum-free medium without the stimulating agent up to 24 h. For comparison, the effect of 24-h exposure to histamine or forskolin is shown. The effect of incubation of CHOrH_2 cells with 100 μM histamine (*filled bars*) or 10 μM forskolin (*open bars*) on [^{125}I]APT binding is expressed as the percentage of [^{125}I]APT binding sites of nontreated cells. Data shown are the mean ± S.E. from 4 experiments. The *asterisks* indicate a significant difference ($p < 0.05$) from control, represented by nontreated cells. *B*, effect of the protein kinase A inhibitor H-89 on histamine- and forskolin-induced H_2 receptor down-regulation. CHOrH_2 cells were incubated for 24 h with histamine (*HA*) or forskolin (*FORS*) in the presence of H-89. The effect of incubation of CHOrH_2 cells with 10 μM H-89 and 100 μM histamine or 10 μM forskolin on [^{125}I]APT binding is expressed as the percentage of [^{125}I]APT binding sites of cells treated with 10 μM H-89 alone. Data shown are mean ± S.E. from 4 experiments. The *asterisks* indicate a significant difference ($p < 0.05$) from control, represented by cells treated with H-89.

mine-induced response was also found to be affected in CHOrH_2Leu^{124}Ala cells (E_{max} in CHOrH_2 cells: 40 ± 4 pmol/well, E_{max} in CHOrH_2Leu^{124}Ala cells: 18 ± 1 pmol/well).

Histamine-induced Down-regulation of Rat H_2 Leu^{124} → Ala Receptors—Long-term exposure (24 h) of CHOrH_2Leu^{124}Ala cells to increasing concentrations of histamine resulted in a dose-dependent reduction of [^{125}I]APT binding sites (Fig. 9*B*). Whereas in CHOrH_2 cells an EC_{50} of 18 ± 6 nM (mean ± S.E., $n = 7$) was observed for histamine, in CHOrH_2Leu^{124}Ala cells histamine induced down-regulation with an EC_{50} value of 288 ± 89 nM (mean ± S.E., $n = 4$). Comparing the histamine-induced cAMP production and H_2 Leu^{124} → Ala receptor down-regulation (Fig. 9*B*), a discrepancy in dose relationships is observed. Almost 40-fold higher concentrations of histamine are required to induce cAMP production, compared to receptor down-regulation. Pretreatment of CHOrH_2Leu^{124}Ala cells for 24 h with 1 μM histamine resulted in a significant degree of H_2 receptor down-regulation (51 ± 2%, mean ± S.E., $n = 4$), whereas no significant cAMP production was observed after 10 min of incubation (Fig. 9*B*). Even after 24 h of incubation of CHOrH_2Leu^{124}Ala cells with 1 μM histamine, no significant increase in cAMP was observed (data not shown). Moreover, even at 0.1 μM histamine, significant H_2 receptor down-regulation was observed.

In the CHOrH_2Leu^{124}Ala cells, the maximal histamine-induced down-regulation was more pronounced (68 ± 4%, mean ± S.E., $n = 4$) than was observed for the CHOrH_2 cells (43 ± 4%, mean ± S.E., $n = 7$). The forskolin (10 μM)-induced H_2 receptor down-regulation was also found to be more pronounced in the CHOrH_2Leu^{124}Ala cells (67 ± 1%, mean ± S.E., $n = 3$) than in CHOrH_2 cells (58 ± 2%, mean ± S.E., $n = 4$).

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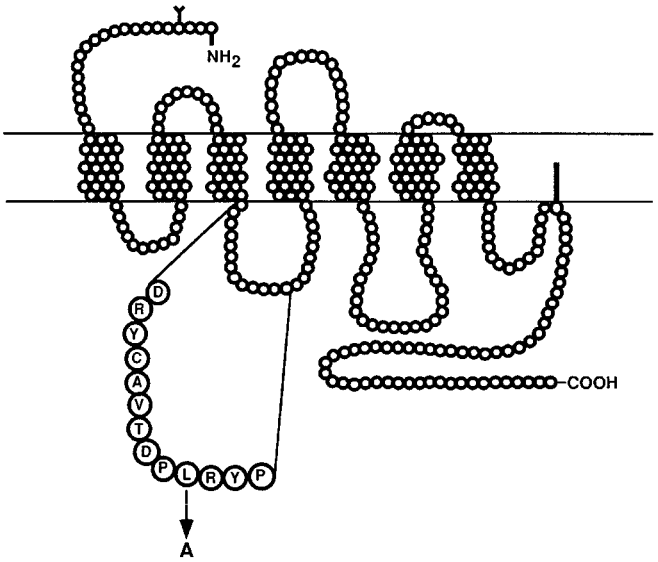

FIG. 7. **Schematic representation of the rat histamine H_2 receptor.** The leucine (*L*) at position 124, located in the second intracellular loop, was mutated to an alanine (*A*) by site-directed mutagenesis as described under "Materials and Methods."

TABLE III
Pharmacological characterization of [^{125}I]APT binding to CHOrH_2 and CHOrH_2Leu^{124}Ala cell membranes

Membranes of CHOrH_2 and CHOrH_2Leu^{124}Ala cells were incubated with 0.3 nM [^{125}I]APT in the presence of the indicated drugs at increasing concentrations. K_i values were obtained from the respective IC_{50} values. Data shown are mean ± S.E. from at least three independent experiments. Values in parentheses indicate the relative densities of the high/low affinity binding site.

Compounds	K_i	
	CHOrH_2	CHOrH_2Leu^{124}Ala
Cimetidine	804 ± 53 nM	766 ± 73 nM
Ranitidine	232 ± 45 nM	173 ± 20 nM
Histamine		
$K_{i,\ high}$	3.5 ± 1.0 μM (39 ± 7%)	
$K_{i,\ low}$	0.24 ± 0.04 mM (61 ± 7%)	0.21 ± 0.02 mM (100%)
+10 μM GTPγS	0.18 ± 0.02 mM (100%)	0.23 ± 0.03 mM (100%)

DISCUSSION

In the present study we have demonstrated that the rat histamine H_2 receptor density in CHO cells is reduced about 50% by long-term exposure to histamine or selective H_2 agonists. Long-term treatment of CHOrH_2 cells with histamine resulted in a time-dependent ($t_{½} \approx 7$ h at a concentration of 100 μM) and dose-dependent (EC_{50} = 18 nM at 24 h of incubation) decrease in the number of H_2 receptor binding sites. Yet, incubation of CHOrH_2 cells with homo- and nordimaprit, two side chain homologues of the H_2 agonist dimaprit with weak H_2 agonistic activity ((23), present study), did not significantly reduce the number of H_2 receptors. These findings show that the observed H_2 agonist-induced down-regulation is a H_2 receptor-mediated process. Long-term exposure of CHOrH_2 cells to histamine resulting in a reduction of H_2 receptor binding sites is paralleled by a decrease of H_2 receptor responsiveness, characterized by a 34-fold shift of the histamine dose-response curve. The observed shift cannot be ascribed to decreased adenylyl cyclase activity as forskolin dose-response curves remained unaffected after long-term histamine exposure.

As was found for the β_2-adrenergic receptor (24), a cAMP-dependent pathway can also regulate the H_2 receptor density. Forskolin, generating cAMP upon addition, time dependently ($t_{½} \approx 7$ h at a concentration of 10 μM) and dose dependently (EC_{50} = 0.3 μM at 24 h of incubation) induced H_2 receptor down-regulation. CTX and IBMX, agents that also elevate intracellular levels of cAMP in CHOrH_2 cells, induced down-regulation of the H_2 receptor as well. Thus, the H_2 receptor does not need to be stimulated by an agonist in order to be down-regulated. This mechanism might be involved in heterologous H_2 receptor down-regulation as previously shown for other GPCRs (see Refs. 4 and 25). The time course of the forskolin-induced decrease of H_2 receptor number in CHOrH_2 cells parallels the time-dependent decrease of H_2 receptors induced by histamine. For both histamine and forskolin, half-maximal H_2 receptor down-regulation is reached after approximately 7 h of incubation. Moreover, the maximum decrease of H_2 receptor numbers induced by forskolin is comparable to the maximum agonist-mediated H_2 receptor down-regulation.

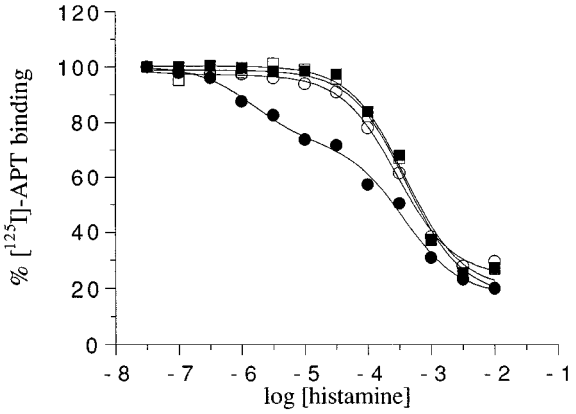

FIG. 8. **Binding of histamine to the wild-type and H_2 Leu^{124} → Ala receptor.** Displacement of binding of 0.3 nM [^{125}I]APT by increasing concentrations of histamine in the presence (*open symbols*) and absence (*filled symbols*) of 10 μM GTPγS in CHOrH_2 cells (*circles*) and CHrH_2Leu^{124}Ala cells (*squares*). Mean values of triplicate determinations of a typical experiment out of at least three are shown.

Agonist-induced receptor down-regulation is a commonly occurring regulatory process of the large family of GPCRs (see for reference reviews, Refs. 4 and 25). Enhanced degradation and/or decreased synthesis of the receptor protein are thought to contribute to receptor down-regulation (4, 25). Agonist-induced down-regulation of GPCRs is often accompanied by a decline of receptor mRNA levels, presumably contributing to the overall reduction in receptor number and responsiveness (26). Indeed, incubation of CHOrH_2 cells with histamine or forskolin resulted in a transient decrease of H_2 receptor mRNA levels (70% reduction) within 4 h, which was followed by a gradual increase of H_2 receptor mRNA to 50% of control mRNA levels in the following hours. The reduced H_2 receptor mRNA levels, 50% of the control levels, at later time points are considered to represent a new steady-state level of receptor mRNA to maintain the down-regulated state of H_2 receptors. The reduction of H_2 receptor mRNA is most likely explained by post-transcriptional events, such as receptor mRNA destabilization. For example, the β_2-adrenergic receptor and thrombin receptor in DDT_1MF-2 smooth muscle cells, the endothelin ET_B receptor in ROS17/2 rat osteosarcoma cells, and also for the β_2-adrenergic receptor and muscarine m1 receptor expressed into CHW and CHO cells, respectively, the decline in receptor mRNA has been ascribed to destabilization of the mRNA (24, 27–30). In the presence of actinomycin D, breakdown of the H_2 receptor mRNA in CHOrH_2 cells was stimulated significantly upon histamine-treated and forskolin-treated compared to non-treated cells. Recently, it was shown that a so-called M_r = 35,000 β-adrenergic receptor mRNA-binding protein, involved in the destabilization of β_2-adrenergic receptor mRNA, also recognizes other GPCR transcripts (29). As such, our observations of H_2 receptor mRNA destabilization fit well in an appar-

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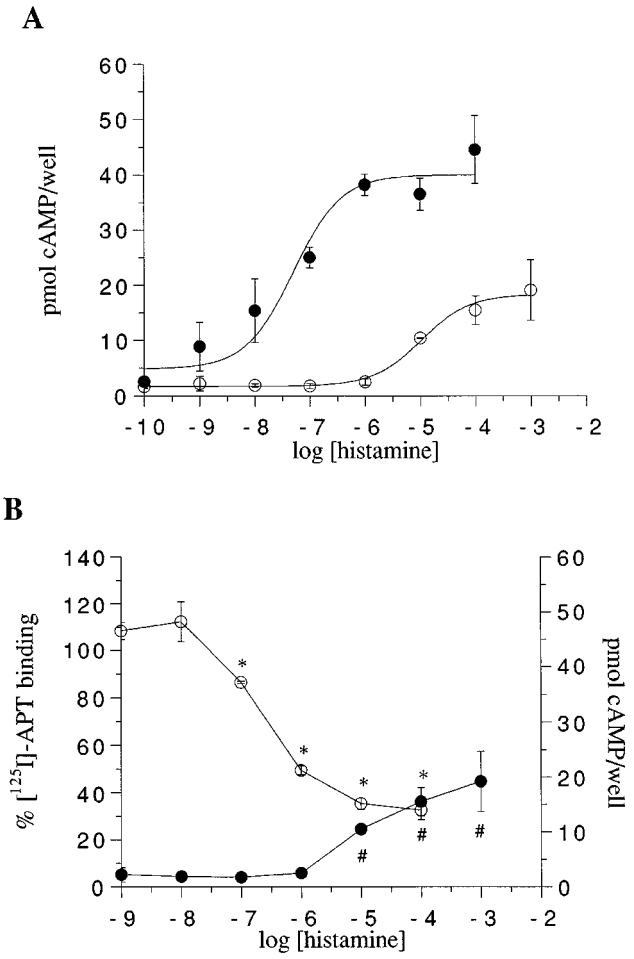

FIG. 9. **Effects of Leu^{124} → Ala mutation on histamine-induced cAMP production and down-regulation.** *A*, dose-dependent increase of the cAMP production by histamine in CHOrH_2 cells (*filled circles*) and CHOrH_2Leu^{124}Ala cells (*open circles*). Cells were incubated with increasing concentrations of histamine for 10 min at 37 °C in DMEM in the presence of 300 μM IBMX and 25 mM HEPES, pH 7.4. The data shown represent the mean ± S.E. for, respectively, 6 and 7 independent experiments. *B*, effects of Leu^{124} → Ala mutation on histamine-induced H_2 receptor down-regulation (*open circles*) and cAMP production (*filled circles*, see also above). CHOrH_2Leu cells were exposed to increasing concentrations of histamine for 24 h, and [^{125}I]APT binding in membranes was measured. The [^{125}I]APT binding is expressed as a percentage of [^{125}I]APT binding measured in nontreated cells. The data shown represent the mean ± S.E. of 4 experiments. The *asterisk* and *number sign* indicate a significant difference ($p < 0.05$) from control, represented by untreated cells and basal cAMP levels respectively.

ently general mechanism of β-adrenergic receptor mRNA binding protein-mediated regulation of GPCR mRNA (29, 31).

For the β_2-adrenergic receptor, the most extensively studied GPCR, receptor down-regulation is ascribed to two pathways: an agonist-dependent, protein kinase A-independent, and a protein kinase A-dependent process (4, 25). Evidence for a protein kinase A-independent pathway was obtained by studies which showed unaffected profiles of β_2-adrenergic receptor down-regulation in mutant S49 mouse lymphoma cells defective in signal transduction components (32–35). Receptor-G_s coupling seems to be important for the process of β_2-adrenergic receptor down-regulation, since defects in this coupling introduced by mutations of the receptor or G_s-protein have lead to impaired β_2-adrenergic receptor down-regulation (33–37). Agents responsible for the elevation of intracellular levels of cAMP, such as forskolin and IBMX, or cAMP analogues, *e.g.* dibutyryl cAMP, were shown to induce β_2-adrenergic receptor down-regulation as well, providing evidence for the existence of cAMP-dependent receptor down-regulation (24, 25, 36). In CHW cells, the time course of the cAMP-promoted down-regulation of the β_2-adrenergic receptor was much slower than the β-agonists-induced down-regulation, suggesting that distinct pathways can lead to down-regulation of the β_2-adrenergic receptor (24). Yet, protein kinase A-dependent phosphorylation of the β_2-adrenergic receptor appears to enhance down-regulation, since receptor mutants lacking protein kinase A phosphorylation sites showed impaired agonist-induced down-regulation (24). Taken together, β_2-adrenergic receptor receptor down-regulation seems to require receptor-G_s coupling for the initial loss of receptor binding sites, while the cAMP-dependent decrease of receptor mRNA levels serves to maintain the down-regulated state by establishing a new steady-state of receptor expression (25). The underlying biochemical mechanisms responsible for each of these events is, however, unclear so far.

In our study on CHOrH_2 cells, comparable time courses and a maximum extent of histamine-induced and forskolin-induced H_2 receptor down-regulation as well as H_2 mRNA down-regulation suggest the involvement of cAMP in the process of agonist-induced H_2 receptor down-regulation. The initial reduction of H_2 receptor mRNA upon histamine or forskolin exposure can, however, not explain the 50% reduction of the H_2 receptor numbers, since relatively short (<4 h) treatments of CHOrH_2 cells with histamine or forskolin followed by a wash-out up to 24 h led to a more pronounced H_2 receptor down-regulation upon histamine than forskolin exposure. Thus, apparently there is no direct link between H_2 receptor mRNA and H_2 receptor expression. Moreover, these data are a first indication that histamine and forskolin induce H_2 receptor down-regulation by different mechanisms. The existence of a cAMP-dependent and cAMP-independent pathway was further corroborated by the fact that the protein kinase A inhibitor H-89 (38) inhibited the forskolin-induced, but not the histamine-induced, H_2 receptor down-regulation. Moreover, recently we have shown that down-regulation of H_2 receptors stably expressed into human embryonal kidney cells (HEK-293 cells) is mediated via cAMP-dependent and cAMP-independent processes as the histamine-induced down-regulation was found to be more pronounced than the forskolin-induced H_2 receptor down-regulation (39).

In order to assess the role of cAMP in the process of agonist-induced H_2 receptor down-regulation in CHOrH_2 cells directly, we constructed a mutant H_2 receptor which showed impaired G-protein coupling. Recently, Moro *et al.* (40) have shown that hydrophobic amino acids within a highly conserved GPCR motif DRY*XX*V(I)*XX*PL (*X* is any amino acid and L is leucine or other lipophilic amino acid) in the second intracellular loop are involved in receptor-G-protein coupling (40). In the H_2 receptor protein, a DRYCAVTDPL sequence is found at an equivalent position of the highly conserved motif (2). Substitution of the Leu^{124} residue by an alanine residue had no effect on H_2 receptor expression nor on H_2 antagonist binding properties. However, the mutation induced a marked impairment of the ability of the receptor to physically couple to its G-protein as assessed by alterations in its agonist-binding parameters (disappearance high affinity binding site, no detectable GTPγS shift). The physical uncoupling of the H_2 Leu^{124} → Ala mutant was paralleled by a functional uncoupling, characterized by an impairment of the histamine-induced cAMP production (160-fold reduction of the EC_{50} value and 55% decrease of the maximal cAMP response). These findings are in agreement with the functional uncoupling reported by Moro *et al.* (40) after mutation of a hydrophobic amino acid at similar position in the muscarine m1, m3, and β_2-adrenergic receptor.

Interestingly, long-term exposure of CHOrH_2Leu^{124}Ala cells to 0.1 μM and 1 μM histamine, concentrations that do *not* elicit

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cAMP production, resulted in a significant reduction of [^{125}I]APT binding sites, indicating that a cAMP-independent pathway is involved in the observed agonist-induced H_2 receptor down-regulation in CHOrH_2Leu^{124}Ala cells. Previous findings in mutant S49 mouse lymphoma cells defective in signal transduction components also showed the existence of cAMP-independent pathways in the agonist-induced β_2-adrenergic receptor down-regulation (32–35). However, it should be noted that the EC_{50} value of histamine-induced H_2 receptor down-regulation was shifted 16-fold to the right for the H_2 Leu^{124} → Ala receptor compared to the wild-type receptor. These data suggest that agonist-induced H_2 receptor down-regulation depends on intact receptor-G-protein coupling. As already stated earlier, previous findings for the β_2-adrenergic receptor have shown that defective receptor-G_s coupling leads to impaired receptor down-regulation (33–37). Remarkably, both the maximum histamine-induced and forskolin-induced down-regulation of H_2 Leu^{124} → Ala receptor were found to be more pronounced than for the wild-type H_2 receptor, suggesting that the mutated receptor has become more susceptible to receptor down-regulation. Although we do not have an explanation for this finding, we hypothesize that the Leu^{124} → Ala mutation induces a conformational change in the second intracellular loop of the H_2 receptor protein, causing an uncoupling from the G_s-protein but also an increase of the accessibility of molecular entities involved in receptor degradation. Recent studies with the parathyroid hormone receptor (41) and β_1-adrenergic receptor (42) support this hypothesis. Small changes in the conformation of intracellular receptor domains have been shown to augment receptor internalization (41, 42). Unfortunately, no data on receptor down-regulation are available for these mutant receptors (41, 42).

In conclusion, for the first time we have demonstrated that the histamine H_2 receptor is down-regulated by prolonged treatment with H_2 agonists. Elevation of cAMP by long-term incubation of CHOrH_2 cells with forskolin, CTX, and IBMX, is also shown to induce H_2 receptor down-regulation. These data suggest the involvement of protein kinase A in the process of H_2 receptor down-regulation and provides a mechanism for heterologous H_2 receptor regulation. Also, H_2 receptor mRNA levels were rapidly down-regulated upon both histamine treatment and forskolin treatment. However, the agonist-induced and forskolin-induced H_2 receptor down-regulation do appear to be differentially regulated, by a cAMP-dependent and cAMP-independent pathway. Substitution of the hydrophobic amino acid leucine 124, located within the highly conserved G-protein coupling motif DRY*XX*V(I)*XX*PL in the second intracellular loop of the H_2 receptor, by an alanine generated a mutant receptor with impaired ability to couple to its G-protein. Interestingly, the H_2 Leu^{124} → Ala mutant receptor was still down-regulated by histamine, at concentrations which showed no increase of cAMP, thereby providing additional evidence for a cAMP-independent pathway in the process of agonist-induced H_2 receptor down-regulation. Thus, H_2 receptor down-regulation appears to be induced by two distinct pathways, a cAMP-dependent and cAMP-independent pathway.

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